Robotic Adherent Cell Injection (RACI) for Characterizing Cell-Cell Communication

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Abstract—Compared to robotic injection of suspended cells (e.g., embryos and oocytes), fewer attempts were made to automate the injection of adherent cells (e.g., cancer cells and cardiomyocytes) due to their smaller size, highly irregular morphology, small thickness (a few micrometers thick), and large variations in thickness across cells. This paper presents a robotic system for automated microinjection of adherent cells. The system is embedded with several new capabilities: automatically locating micropipette tips; robustly detecting the contact of micropipette tip with cell culturing surface and directly with cell membrane; and precisely compensating for accumulative positioning errors. These new capabilities make it practical to perform adherent cell microinjection truly via computer mouse clicking in front of a computer monitor, on hundreds and thousands of cells per experiment (vs. a few to tens of cells as state-of-the-art). System operation speed, success rate, and cell viability rate were quantitatively evaluated based on robotic microinjection of over 4,000 cells. This paper also reports the use of the new robotic system to perform cell-cell communication studies using large sample sizes. The gap junction function in a cardiac muscle cell line (HL-1 cells), for the first time, was quantified with the system.

I. INTRODUCTION

Intercellular communication is a critical part of cellular activities and coordinates cell functions [1]. Disorders of intercellular communication are responsible for diseases such as cancer [2], autoimmunity [3], and diabetes [4]. A standard technique for measuring intercellular communication is based on monitoring transfer of fluorescent molecules from an individual cell to adjacent cells through functional gap junctions (see Fig. 1). The quantitative measurement of dye transfer requires injection of fluorescent molecules into single cells. In present gap junction testing experiments, only a few or significant data, for instance, for testing drug molecules on operation, posing a practical hurdle in attaining statistically significant data, for instance, for testing drug molecules on the alteration of gap junction function [5].

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Manually manipulating single cells is tedious and time-consuming and has high skill requirements. In manual operation, a skilled operator must dexterously control multiple devices such as micromanipulator, pump, and microscope stage. Robotic cell manipulation technologies progressed significantly over the past decade. The vast majority of demonstrated robotic systems focused on the manipulation of suspended cells (i.e., oocytes and embryos) [6]–[11]. Fewer attempts were made to automate the injection of adherent cells.

Most mammalian cells (e.g., cancer cells and cardiomyocytes) adhere to a culturing surface. Different from large suspend cells (e.g., mouse oocytes ~100 µm; zebrafish embryos ~1 mm), adherent cells are smaller in size and highly irregular in morphology (vs. spherical for oocytes/embryos), making robust pattern recognition difficult and automation challenging [12]. Additionally, adherent cells are only a few micrometers thick and vary significantly in thickness, posing more stringent requirements in robotic positioning.

A few joystick-based systems were demonstrated to assist operators for adherent cell microinjection [13], [14]. Long training, low success rates, and poor reproducibility make these systems incapable of injecting more than tens of cells per experiment. For example, the most popular commercial system for adherent cell injection is Eppendorf’s InjectMan system that requires an operator to look into the eyepieces of a microscope and perform microinjection by controlling...
The robotic micromanipulator and X-Y stage are cooperated in this system using well calibrated air pressure to deliver aqueous solution into either the cytoplasm or nucleus of a cell. As shown in Fig. 2(a) and (b), the system consists of a standard inverted microscope (Nikon TE2000-S, Nikon Microscopes) and a motorized X-Y translational stage (ProScan, Prior Scientific, Inc.). The X-Y stage has a travel range of 75 mm along both axes with a resolution of 0.01 μm, a maximum speed of 5 mm/s, and a repeatability of ±1 μm. A CMOS camera (acA1300-32gm, Basler) is connected to the microscope for visual feedback. A glass micropipette, laser pulled to have an outer diameter of 500 nm and inner diameter of 300 nm, is mounted to a 4 degrees-of-freedom (DOF) DC-driven micromanipulator (MX7600, Siskiyou, Inc.) that has a travel range of 20 mm and a 0.1 μm positioning resolution along each axis. A host computer executing computer vision microscopy and motion control algorithms controls all hardware.

The robotic micromanipulator and X-Y stage are cooper-
ately controlled for positioning the micropipette along the XYZ axes and positioning cells in the XY plane, respectively. The overall control architecture of the system is summarized in Fig. 2(c). Microscopy visual feedback is used to control the positioning of the micromanipulator and X-Y stage, forming an image-based visual servo control system.

When a micropipette is mounted on the system, the system detects the micropipette tip’s position and automatically moves it to the center of the field of view [see Section III-A]. The system is capable of detecting the contact in two modes, either on cell culturing surface or directly on cell membrane [see Section III-B&C], to determine the relative vertical distance between the micropipette tip and the cells in the Z direction. After the injection location (inside cytoplasm or nucleus) on each cell is selected via computer mouse clicking, the system controls the micropipette to deliver a precise volume into each cell, and completes the injection of all selected cells following a shortest path. The system then controls the X-Y translational stage to bring cells in the next field of view under microscopy imaging for injection.

III. KEY METHODS

A. Locating Micropipette Tip

When a micropipette tip is mounted on the micromanipulator, the system automatically locates the micropipette tip and moves it to the center of the field of view. This process involves two main steps: micropipette detection and autofocus adjustment.

In the detection step, the micropipette is swept in the X-Y plane for detecting its presence in the field of view. In some cases, the micropipette tip cannot be detected when it is far away from the focal plane. When this occurs, the system moves the focal plane up by controlling the focus adjustment motor, and the X-Y sweep is then repeated. During the sweep of the micropipette, motion history images are generated and used for detecting the presence of the micropipette. The motion history image method is discussed in Section III-C.

In the autofocus step, the system uses the normalized variance method [17] to calculate the focus measure. The autofocus step consists of coarse and fine focus adjustments. Coarse focus adjustment moves the focal plane in a large step size until the entire image produces a maximal focus measure value. In fine focus adjustment, a recursive quad-tree autofocusing method is used to accurately focus on the micropipette tip with smaller step sizes. After the focus adjustments, the in-focus micropipette tip is moved to the center of the field of view through closed-loop visual servoing under a low magnification objective and then under a high magnification objective. The detailed methods for detecting and tracking micropipette tips are described in [18].

B. Contact Detection on Cell Culture Surface

In order to inject materials into a cell, the relative vertical distance between the micropipette tip and the cell along the Z-direction must be accurately detected. Without the inclusion of an extra sensor (e.g., tactile or force sensors), which increases hardware complexity [19], two modes of computer vision-based contact detection algorithms are developed to accurately determine the relative heights of the micropipette tip and adherent cells. This section describes the first mode, in which the system detects the initial contact of micropipette tip on the cell culturing surface that is protein coated (vs. bare surface).

1) Empty Region Detection: When cell confluency is lower than 80%, empty areas on the culturing surface can be detected and used for contact detection. The system divides the field of view into sub-regions, the size of which is 100 pixels×100 pixels. The normalized variance of the pixel value, $F$, is calculated for each region to represent its smoothness, according to

$$ F = \frac{1}{W \cdot H \cdot \mu} \sum_{W} \sum_{H} (I(x,y) - \mu)^2 $$

where $W$ and $H$ are the region width and height, $I(x,y)$ is the pixel intensity at the point $(x,y)$, and $\mu$ is the average region intensity. This algorithm effectively compensates for differences in average intensity among different regions. The region with the lowest variance value is considered empty and used for performing contact detection.

2) Contact Detection via Three-Dimensional Motion: The system moves the detected empty region in the X-Y plane until it is directly under the micropipette tip [Fig. 3(a)&(c)]. The micropipette tip is then lowered by the micromanipulator along the Z direction. When the micropipette tip contacts the bare surface of a Petri dish, further vertical movement of the micropipette tip induces horizontal motion [20]. However, in cell culturing, the Petri dish surface is usually coated with gel or protein (e.g., collagen). The coating makes it difficult for the micropipette tip to ‘slide’ horizontally (it ‘stabs’ directly into the coated layer). Therefore, in this system, the X-Y stage is also servoed simultaneously to move along the direction in which micropipette tip enters the field of view, while the micropipette tip is being lowered by the micromanipulator. The X-Y stage’s motion enables the reliable generation of the pipette tip’s horizontal ‘sliding’ motion [Fig. 3(b)&(d)], resulting in reliable contact detection on protein-coated cell culture surfaces.

![Fig. 3. Detection of contact on cell culture surface. (a) Micropipette tip is lowered towards the cell culture surface while the X-Y stage moves along the X axis from left to right. (b) Micropipette tip slides horizontally when it contacts the culture surface. (c)(d) Schematic side view of (a)(b).](image-url)
C. Contact Detection on Cell Membrane

When cell confluency is high, empty regions on the culture surface becomes unavailable; hence, the system performs contact detection by detecting direct contact of the micropipette tip on the cell membrane [see Fig. 4(a)]. After the operator selects a cell via computer mouse clicking, the system moves the cell to the micropipette tip position in X-Y plane and then moves the micropipette tip downwards along the Z axis. When the micropipette tip contacts the cell membrane, the cell is deformed and a subtle motion appears around the contact point. To detect this subtle motion, we developed an algorithm based on motion history images (MHI). The MHI-based method enhances motion representation by accumulating cell deformation over a period of time.

A region of interest (ROI) is first obtained around the micropipette tip [Fig. 4(b)]. Within the ROI, the image is

\[ I(x, y, t) = b(x, y) + m(x, y) + n(x, y) \]  

(2)

where \( b(x, y) \) is the static background; \( m(x, y) \) is the moving object; \( n(x, y) \) is the background noise; and \( t \) is time. The moving object is extracted via taking the difference between consecutive frames.

\[ D(x, y, t) = I(x, y, t+1) - I(x, y, t) = M(x, y, t) + N(x, y, t) \]  

(3)

where \( M(x, y, t) \) is the motion region; \( N(x, y, t) \) is noise; and \( D(x, y, t) \) is the difference image containing the moving object and background aberration due to motion and noise. Background aberration can lead to incorrect detection (e.g., motion ambiguity and distortion) and make detection fail when object motion speed is low.

In our system, the motion region \( M(x, y, t) \) contains motion from both the lowering micropipette and cell deformation due to micropipette-cell contact. Hence,

\[ M(x, y, t) = M_p(x, y, t) + M_c(x, y, t) \]  

(4)

where \( M_p(x, y, t) \) is the micropipette lowering motion, and \( M_c(x, y, t) \) is the motion from cell deformation. When the micropipette is moved along Z axis during contact detection, the tip’s location in the X-Y plane, \((x_p, y_p)\), is constant, and the lowering motion results from the defocusing phenomenon. Since a defocusing blurred image is equivalent to an exponential decay function [21], the micropipette lowering motion can be described as

\[ M_p(x, y, t) = M(x_p, y_p, t_0) e^{-\lambda t} \]

where \( M(x_p, y_p, t_0) \) is the initial micropipette’s motion before cell contact occurs. Thus, motion from cell deformation is

\[ M_c(x, y, t) = M(x, y, t) - M(x_p, y_p, t_0) e^{-\lambda t} \]  

(5)

Since the micropipette is controlled to move downwards at a constant speed \( v \), its Z-displacement is proportional to time \( t \). Hence, the cell deformation term can also be expressed as a function of Z-displacement,

\[ M_c(x, y, t) = M_c(x, y, Z/v) \]

After removing micropipette tip’s motion, the noise term \( N(x, y, t) \) in the difference image can be removed by a Gaussian low-pass filter and image binarization. The binary image representing cell deformation-caused motion is

\[ \Psi(x, y, t) = \begin{cases} 1 & \text{if } M_c(x, y, t) > \xi \\ 0 & \text{otherwise} \end{cases} \]  

(6)

The motion history image is

\[ H_r(x, y, t) = \begin{cases} \tau & \text{if } \Psi(x, y, t) = 1 \\ \max(0, H_r(x, y, t-1) - \gamma) & \text{otherwise} \end{cases} \]  

(7)

where the duration \( \tau \) determines the temporal extent of the movement, and \( \gamma \) is the decay parameter. The subtle cell deforming motion is greatly enhanced by adding the motion history information [see Fig. 4(d)].

The sum of pixel values in MHI, \( s(H) \), is calculated as a measure to determine the contact of cell membrane. If \( s(H) \) is above a threshold value \( \delta \), the micropipette tip is considered as contacting cell top membrane. The threshold \( \delta \) is calculated dynamically by analyzing MHIs in which the micropipette tip has not contacted cell surface.

\[ \delta = \bar{s} + a \cdot \sigma(s) \]  

(8)

where \( \bar{s} \) is the average of MHIs’ sum of pixel values before cell contact occurs; \( \sigma(s) \) is the standard deviation; and \( a \) is a preset parameter which is experimentally determined. By adding \( a \cdot \sigma(s) \) to the mean value, the threshold \( \delta \) effectively rejects those MHIs that do not contain cell deformation. In addition, the motion gradient of the MHI is also used to avoid false positive detection. The motion gradient is computed by convolution with Sobel filters to yield spatial derivatives, \( F_x(x, y) \) and \( F_y(x, y) \). The orientation of the motion gradient at each pixel is

\[ \Phi(x, y) = \arctan \frac{F_y(x, y)}{F_x(x, y)} \]  

(9)

As the micropipette tip touches the cell membrane, deformation occurs around the contact point. Accordingly, the motion gradients in MHI converge to the contact point.
D. Robotic Microinjection

In this new robotic system, an operator performs every step throughout the microinjection process via computer mouse clicking. The operator readily selects injection locations (i.e., cell nucleus or cytoplasm) since the cell nucleus and cytoplasm are distinct on a computer monitor. The operator can select a single cell or multiple cells to inject within the field of view. During computer mouse clicking, cell templates centered at the mouse clicking positions are extracted. The templates are used for template matching to provide position feedback. After performing contact detection, the system moves the micropipette tip close to the first target cell and inserts the micropipette into the target cell along the tilting axis at the maximal speed. Injection depth was set to be either two micrometers above the cell bottom surface (i.e., detected by contact on cell membrane) or two micrometers below the cell top surface (i.e., detected by contact on dish substrate). The pressure source is then triggered to apply a positive pressure to deposit the preloaded material into the cell. Material deposition results in a “shock wave” motion inside the cell. At the injection location [see Fig. 5(b)], the volume of injected materials is precisely controlled by the injection pressure magnitude and width. Although target cells are selected in a random order by the operator, the system injects all cells along the shortest path. The system then moves to the next field of view to continue the injection of more cells. The locations of injected cells are all recorded by the system, permitting time-lapsed imaging of every injected cell.

IV. RESULTS AND DISCUSSION

A. Performance of Locating Micropipette Tip

To evaluate the system’s performance of automatically locating micropipette, micropipette tip’s initial distance to the culture surface was divided into five groups. The task of locating micropipette tips in the five groups was also conducted manually by three skilled micromanipulation operators.

B. Injection Speed

Injection speed of the system was evaluated by injecting three different adherent cell lines: HeLa cells, HEK293 cells, and HL-1 cells. The number of injected cells and time consumed are summarized in Table I. The injection time summarized in Table I is the total experimental time for all steps including locating micropipette tip (after replacement of a new micropipette due to tip damage or clogging), contact detection, cell selection, and injection. The results from the injection of over 4,000 cells show that the injection speed of the robotic system is consistent across different adherent cell lines. The average injection speed is 22.7 cells/min, which enables users to inject over a thousand of adherent cells within one hour.

C. Injection Success Rate and Cell Survival Rate

In order to evaluate the injection success rate and cell survival rate, a Dextran-Rhodamine fluorescent dye was injected into the cytoplasm or nucleus of HeLa cells. The Dextran-Rhodamine dye was chosen because it is highly water-soluble and membrane-impermeable. Therefore, only the cells that are successfully injected with Dextran-Rhodamine are able to reveal red fluorescent color. The system permits material deposition into the cytoplasm [Fig. 5(e)] or into the cell nucleus [Fig. 5(d)]. When the deposition target (i.e., either cytoplasm or nucleus but not both) reveals strong red fluorescent signals, injection was considered successful. Experimental results summarized in Table II show that the success rates for cytoplasmic (n=1,245) and nuclear (n=1,036) injection were comparable (95.2% and 97.5%).

![Image of cell nucleus and cytoplasm](image-url)
Cell post-injection viability was evaluated by using a cell viability assay kit (Viability/Cytotoxicity Kit, Life Technologies) after 30 min post-injection incubation. The measured cell survival rate after cytoplasmic injection was 97.2% and after nuclear injection was 96.5%.

D. Characterization of Cell-Cell Communication

We used the robotic system to perform dye transfer experiments for measuring gap junctional intercellular communication (GJIC). In the dye transfer experiments, a membrane-impermeable HPTS (8-Hydroxypyrene-1,3,6-Trisulfonic Acid) fluorescent dye mixing with Dextran-Rhodamine dye was injected into a cell. HPTS fluorescent dye was chosen because of its small molecule size and membrane-impermeable property. The small HPTS molecule (MW=524.37 Da) can be transferred from the injected cell to adjacent cells through only gap junctions. In contrast, the Dextran-Rhodamine molecule, because of its large molecule size (MW=10,000 Da), cannot pass through gap junctions. Therefore, only the injected cell reveal a red fluorescent signal, while the injected cell as well as a number of adjacent cells reveal green fluorescent signal (see Fig. 6). The exact number of neighboring cells that can reveal green fluorescent signal is determined by the capability of the cells’ gap junctions. Thus, gap junctional intercellular communication was quantitatively investigated by measuring the dye transferred cell number (i.e., the number of cells uptaking fluorescent molecules from the injected cell through gap junctions) and dye transferred distance (i.e., the furthest distance that the fluorescent dye can be transferred).

Gap junctional intercellular communication was investigated on three cell lines (i.e., Hela cells, HEK293 cells, and HL-1 cells). These cell lines are known to have absent, moderate, and high expression of gap junctions, respectively. We also used 18-α glycyrrhetinic acid (18-αGA) to treat HL-1 cells as a demonstration of quantifying drug effect on regulating gap junction function. It is known that 18-αGA inhibits the role of GJIC in fibroblast growth [22], myoblast fusion [23], and trophoblast proliferation [24]. In our experiments, the robotic system was used to assess GJIC of three experiment groups with different doses (25, 50, or 100 µM) of 18-αGA treatment.

Two control groups were also included. Control group 1 (0 µM in Fig. 7(b)) had no 18-αGA. Since the 18-αGA stocking solution was dissolved in DMSO, a second control group (DMSO in Fig. 7(b)) with only DMSO treatment was also examined.

The dye transfer distance was measured to quantitatively characterize the GJIC for the three cell lines. The histogram of dye transfer distance for the three cell lines shown in Fig. 7(a) reveals that HL-1 cells have higher GJIC than the other two cell lines. HL-1 is a cardiac muscle cell line. The strong GJIC of HL-1 cells is physiologically necessary for impulse propagation in cardiac tissue [25]. The impulse signal is passed efficiently through gap junctions, allowing the cardiac tissue to contract at the same tandem. In contrast, Hela cells, a cancer cell line derived from cervical cancer cells, express little GJIC, confirming they do not pass signals via gap junctions for cell coordination. Limited GJIC results in poor inhibition of cancer cell mitosis, causing tumor formation [26].

The experimental data in Fig. 7(b) shows that the number of HL-1 cells that uptook fluorescent dye from the injected cells decreased significantly with a higher dose of GJIC inhibitor. The results show that 100 µM of 18-αGA effectively blocked almost all the gap junctions. There is no significant difference
between the two control groups of HL-1 cells (i.e., the first two bars in Fig. 7(b)).

E. Discussion

Microinjection of adherent cells permits the direct insertion of foreign materials (e.g., DNA/RNA, fluorescent dyes, quantum dots etc.) into single cells. It is a decades-old technology that is still widely used in cell biology for testing cell-cell communication, studying intracellular behavior, and gene transfection [27].

Manual microinjection of adherent cells and existing robotic system prototypes are limited to the injection of a few to tens of cells per experiment at best. Our new robotic system described in this paper is the first system capable of performing microinjection on hundreds and thousands of cells per experiment. The system is embedded with strong automation capabilities in every step of operation, enabling an operator to perform the entire microinjection process via mouse clicking in front of a computer monitor. Training of a user with no skills in microinjection takes 10–15 minutes, and after a few hours' operation, the user becomes highly proficient at operating the system to perform adherent cell microinjection with high success rates.

V. CONCLUSION

This paper described a robotic adherent cell injection system equipped with several key technologies that were recently developed. These new techniques include automatically locating micropipette tips, robustly detecting the contact of micropipette tip with cell culture surface and directly with cell membrane, and precisely compensating for accumulative positioning errors. The high degree of automation of the system enables users with no microinjection training to perform large-scale cell injection with high success rates. System operation speed, success rate, and cell viability rate were quantitatively evaluated. Dye transfer experiments were conducted on different cell lines to establish a powerful assay for characterizing cell gap junction functions.

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